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Lumcorin: A leucine-rich repeat 9-derived peptide from human lumican inhibiting melanoma cell migration

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ABSTRACT

We previously showed that lumican decreases melanoma progression. The aim of the present study was to determine the active sequence of the lumican core protein responsible for the inhibition of melanoma cell migration. Using different recombinant and synthetic peptides derived from lumican, we localized an active site in the leucine-rich repeat 9 domain of the lumican core protein. We propose the name lumcorin (fragment of *lum*ican *cor*e prote*in*) for the active peptide derived from this site. Lumcorin was able to inhibit melanoma cell migration in vitro.

1. Introduction

Lumican is an extracellular matrix (ECM) protein present in normal adult skin as a glycoprotein with a 37 kDa core protein. It was first identified in the cornea, where it is expressed as a proteoglycan with keratan sulfate chains [1]. Lumican belongs to the small leucine-rich proteoglycan (SLRP) family which also includes decorin, biglycan and fibromodulin [2]. A signature characteristic for the SLRP family is the leucine-rich repeat (LRR) domains which consists of internal tandem repeats rich in leucine. These repeats contain the 11-amino acid hallmark motif LxxLxLxxNxl/L [3]. Lumican contains 11 LRR motifs [4]: six of them are constituted by the exact sequence LxxLxLxxNxL and five by a close sequence. There is a correlation between internal repeats and the "banana-shaped" tertiary structure of the SLRP, thought to be involved in protein–protein interactions [5]. Some LRR of SLRP have biological activities: LRR 5 and 6 from decorin and LRR 11 from fibromodulin are involved in collagen type I binding [6,7]. Moreover, the decorin LRR 5 shows anti-angiogenic properties [8,9]. Like decorin and fibromodulin, lumican contributes to collagen fibrillogenesis [10]. Knock-out mice for lumican have a fragile dermis due to irregular fibrillar meshwork. In addition to its role in collagen fibrillogenesis, lumican possesses anti-tumor activity. It inhibits melanoma progression in vivo and in vitro [11–13]. In breast cancer, a low expression of lumican correlates with poor outcome of invasive carcinoma [14].

So far, the specific amino-acid sequence of the lumican core protein responsible for inhibiting lumican-dependent tumor cell migration has not been determined. The aim of the present study was to identify the active amino-acid sequence of lumican. We demonstrated that a peptide from the LRR 9 domain of human lumican inhibits melanoma cell migration. We propose the name of lumcorin (fragment of *lum*ican *cor*e prote*in*) for this anti-migratory peptide.

2. Materials and methods

2.1. Peptide synthesis

The peptides corresponding to LRR motif lumican sequences LQHNRLKEDAVS (peptide 5), VSLLTLYLDNNKISNIP (peptide 7), NALQYLRLSHNELADSG (peptide 8), SSLVELDLSYNKLKNIP (peptide 9, lumcorin), SYSKIKHLRLDGNRISE (peptide 11) and the scrambled peptide LPSVSILEKLYNNLSKD (peptide 9 SCR), were obtained from

Abbreviations: BSA, bovine serum albumin; ECM, extracellular matrix; LRR, leucine-rich repeat; SLRP, small leucine-rich proteoglycan

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GenScript Corporation (Piscataway, NJ, USA). Peptides were dissolved in 18 mM acetic acid.

2.2. Cell culture

Human melanoma cell lines A375 (CRL-1619), HT144 (HTB-63™) and murine melanoma B16F1 (CRL-6323) were obtained from the American Type Culture Collection (ATCC) and cultured as recommended by supplier.

2.3. Cell viability and apoptosis assay

For measuring cell viability, cells (15×10^3 per well) were plated into 96-well plates. At the 2nd day, peptide 9 or 9 SCR (100 μM) were added into the wells. The toxicity of the peptides was then measured using 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT).

For apoptosis assay, cell nuclei were stained with Hoechst 33342 (1 μg/ml) 24 h after incubation of the cells with peptide 9 or 9 SCR. Doxorubicin (2 μM), a pro-apoptotic drug, was used as positive control [15]. Nuclear morphology (nuclear fragmentation) was visualized with an inverted fluorescence microscope (Axiovert 200M, Zeiss, Oberkoken, Germany).

2.4. Expression and purification of recombinant lumican core protein and its fragments

Recombinant human lumican core protein (37 kDa) was produced as previously described [12]. Recombinant lumican fragment, L1–9 (Gly#15-Leu#266), was obtained after digestion of the human lumican cDNA by KpnI and HindIII and cloned into the multiple cloning site of PQE-30 vector (Qiagen, Courtaboeuf, France).

L1–6 fragment (Gly#15-Ser#206) was obtained using 5′-gagctcggtaccagtggccagtactat-3′ forward and 5′-tggtgaaagcttagagacagg gagaccaga-3′ reverse primers. The obtained cDNA fragment was cloned into the KpnI/HindIII site of PQE-30. The sequence of both constructs was checked by sequencing. The recombinant lumican and fragments were purified on Ni-NTA resin superflow affinity chromatography via their 6× Histidine tag as already described [12]. Lumican core protein and fragments were dissolved in 18 mM acetic acid.

2.5. Western immunoblotting

The rabbit polyclonal antibody raised against the synthetic human lumican peptide (17 amino acids: YLDNNKISNIPDEYFKR), used in our previous works [11], does not recognize L1–6 fragment. Immunoreactive serum raised against human lumican was prepared after three intra-dermal injections of human lumican core protein every 3 weeks in a rabbit. First injection contained complete Freund's adjuvant and the boosters incomplete Freund's adjuvant. The serum was purified through DEAE-Affi-gel Blue gel column (Bio-Rad Laboratories, Marnes-la-Coquette, France). The specificity of the purified serum was checked by Western immunoblotting on total protein extracts from bovine cornea and on human recombinant lumican core protein. The immunoreactivity efficiency of this immunopurified serum was compared to the rabbit polyclonal antibody raised against the synthetic human lumican peptide used in our previous works.

Following electrophoresis, recombinant human lumican core protein and fragments were transferred from the polyacrylamide gels to nitrocellulose by electroblotting. The membranes were soaked in TBS-T solution (0.005% Tween 20, 20 mM Tris and 140 mM NaCl, pH 7.6) containing 5% bovine serum albumin (BSA) for 2 h. After washing, the membranes were incubated with the immunopurified serum raised against the recombinant human lumican at a final dilution of 1:500 overnight at 4 °C with constant agitation. The membranes were washed with TBS-T and probed with 1:10 000 dilution of a goat anti-rabbit secondary antibody conjugated to horseradish peroxidase in a solution of 1% BSA in TBS-T for 45 min at room temperature. After washing in TBS-T, the complexes were revealed by the Amersham™ ECL Chemiluminescence Detection kit (GE Healthcare, Buckinghamshire, UK).

2.6. Cell migration assay

Cell migration stimulated by serum gradient were performed in Transwell® chambers (Greiner Bio-one, Courtaboeuf, France) as previously described [13]. Transwell® chambers were coated either with 10 μg of recombinant human lumican core protein or equimolar amounts of BSA or 10 μg of type I collagen. Cells (5×10^4) in 100 μL medium supplemented with 0.2% BSA were placed into the upper chamber of the Transwell® device. Six hundred and fifty microliters of medium containing 2% BSA and 10% FBS were placed into the lower chamber. To investigate the effect of the different synthetic peptides, cells were pre-incubated for 2 h with peptides 5, 7, 8, 9, 11 or 9 SCR (100 μM) before being added to the upper chamber of the Transwell®. After 24 h of incubation, the cells were fixed with methanol and stained with crystal violet. The cells remaining in the upper chamber were removed by cotton swab. Migrated cells, on the lower side of the filter, were counted in five random fields using the Cell Counter plugin of the ImageJ software (Rasband, W.S., ImageJ, U.S. National Institutes of Health, Bethesda, Maryland, USA, http://rsb.info.nih.gov/ij/, 1997–2008).

2.7. Homology modeling of lumican

The 3D structure of lumican was obtained using Swiss-Pdb Viewer software [16] from the crystal structure of decorin and biglycan (PDB # 1xku and PDB # 2ft3) as template.

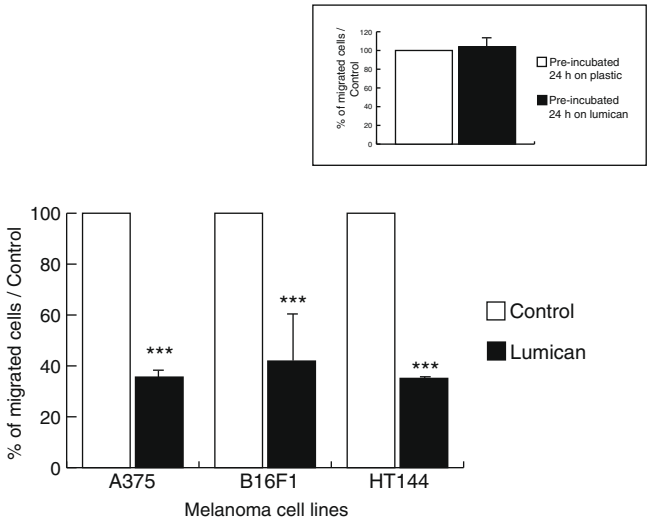

Fig. 1. Reversible inhibition of melanoma cell migration by lumican core protein. Recombinant lumican core protein inhibits melanoma cell migration. Cells (5×10^4) were seeded on Transwell® membranes coated with lumican (10 μg) or equimolar amount of BSA (controls) and incubated for 24 h at 37 °C. The cells were stained with crystal violet and were counted in five random fields. Insert: A375 cells were pre-incubated on plastic or on lumican coating for 24 h. Cells were then detached and seeded on Transwell® membranes without any coating. The percentage of migrated cells was quantified after 24 h of migration. ∗, significantly different from the control (**$P < 0.01$, ***$P < 0.001$).

2.8. Statistical analysis

Results were expressed as the means ± S.D. of three replicates and are representative of three independent experiments. Statistical significance was assessed, by the comparison of each group to the control group, by unpaired Student's test, with $P < 0.05$ being considered as significant. Analysis of variance (ANOVA) was performed using the Fisher's test.

3. Results

3.1. The human lumican core protein inhibits melanoma cell migration in a reversible manner

To study the effect of the lumican core protein on melanoma cell migration, we selected three melanoma cell lines: Human melanoma A375 and HT144 cells and murine melanoma B16F1 cells. Lumican significantly inhibited the three melanoma cell line migration in Transwell® devices (Fig. 1). The inhibitory effect of lumican was reversible: the migration of A375 cells, pre-incubated for 24 h on a lumican-coated plate, was not affected when these cells were no longer in contact with the lumican core protein (Fig. 1, insert).

3.2. Identification of an active fragment within lumican core protein able to inhibit melanoma cell migration

In order to identify the sequence responsible for the inhibition of tumor cell migration within the lumican core protein, two recombinant fragments of lumican: L1–9 (30.5 kDa, containing the LRR 1–9) and L1–6 (23.9 kDa, containing the LRR 1–6) were produced (Fig. 2A and B). Transwell® membranes were coated with these fragments, or the complete lumican core protein, in equimolar amounts. As shown in Fig. 2C, L1–9 fragment inhibited A375 cell migration by 60% ($P < 0.01$), whereas L1–6 fragment had no effect, indicating that an active site for migration inhibition was present within the LRR 7–9 domain of lumican.

3.3. The LRR 9 motif inhibits melanoma cell migration

Lumican, decorin, biglycan and fibromodulin belong to SLRP family and have similar biological activities, particularly anti-tumor properties [17,18]. A sequence with an important function such as the inhibition of cell migration, should be conserved among the SLRPs. Thus, we aligned the amino acid sequences of these four SLRPs and focused on the LRR 7–9 region (Fig. 3A). The sequence alignment showed 54% (6/11) of conserved amino acids in the LRR 9 motif, whereas 27% only (3/11) were conserved

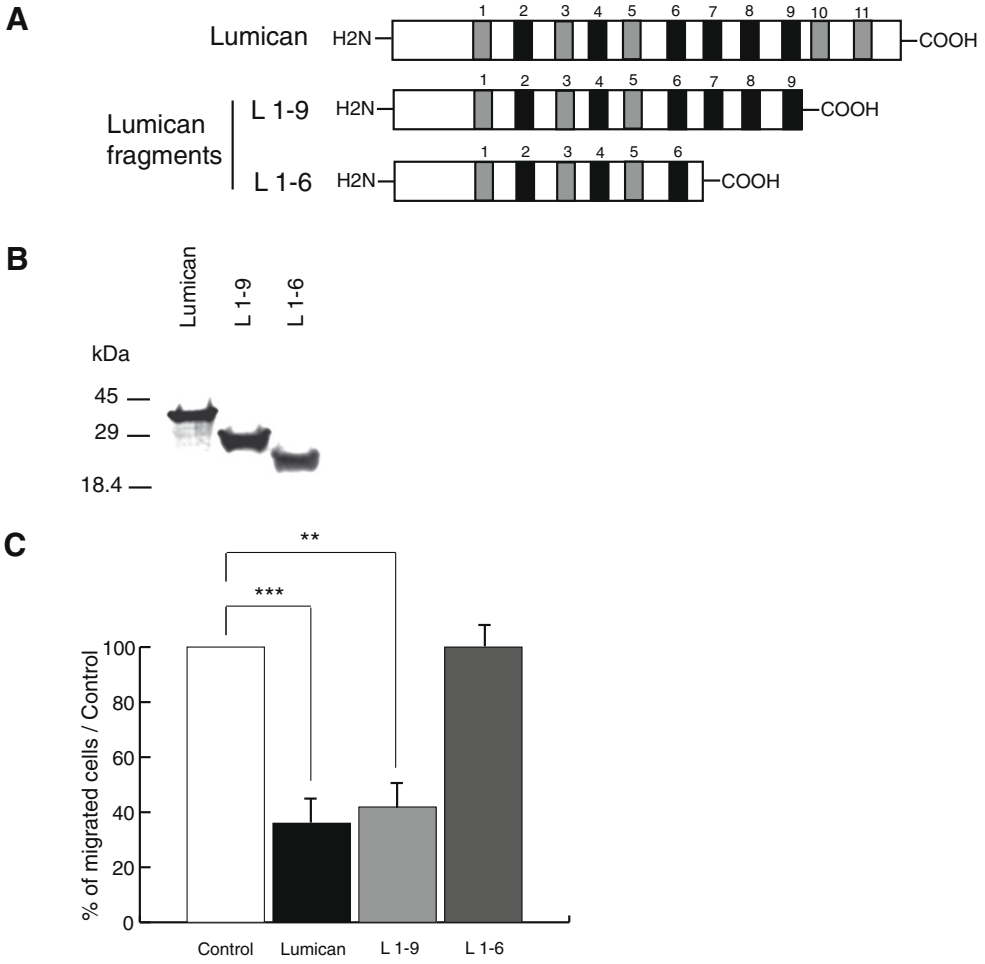

Fig. 2. Identification of an active domain within the lumican core protein able to inhibit melanoma cell migration. (A) Schematic representation of the human recombinant lumican core protein and its fragments used for this study. The LxxLxLxxNxL motifs are represented by the numbered black (consensus) and grey (partial consensus) boxes. (B) Western immunoblotting detection of the lumican core protein and the L1–6 and L1–9 fragments. The position migration of size markers are depicted on the left margin. (C) Effect of the recombinant lumican fragments on A375 melanoma cell migration. The migration assay was processed as described in Section 2, using Transwell® membranes coated with BSA (control), lumican or lumican fragments. Analysis of variance (ANOVA) performed with the Fisher's test confirmed that the four groups of data were significantly different for one group to another one ($F^3_{56} = 9.56$, $P < 0.001$). ∗, significantly different from the control by unpaired Student's test (**$P < 0.01$, ***$P < 0.001$).

in both LRR 7 and 8 motifs. Therefore, the LRR 9 motif was considered as a good candidate to lead the anti-migratory effects of the complete lumican core protein. Accordingly, a synthetic peptide (peptide 9), corresponding to the LRR 9 motif with its flanking sequences was studied for its anti-migratory properties. As controls, we also used two other peptides (peptide 5 and peptide 11) chosen outside the active LRR 7–9 fragment and corresponding to the LRR 5 and LRR 11 motifs of human lumican, respectively. The localization and amino-acid sequence of these peptides on the lumican core protein are presented in Fig. 3B. Homology modeling showed that the amino acid residues of peptides 8, 9 and 11 were located in the concave side of lumican, whereas most of residues from peptide 5 were in the convex side (Fig. 3C).

The three synthetic peptides (5, 9 and 11) were tested at a 100 μM concentration on A375 cell migration in Transwell® devices. As shown in Fig. 4A, peptide 9, but not peptides 5 or 11, significantly decreased A375 cell migration compared to the control, with a dose-dependent response (correlation coefficient $r = 0.994$, $P < 0.001$). Thus, a concentration of 100 μM for the peptide 9 was chosen for the following experiments. None of the studied peptides showed any cytotoxic or pro-apoptotic effects, as appreciated by the MTT test or Hoechst staining, respectively (data not shown). Migration assays with other melanoma cell lines (Fig. 4B) showed that peptide 9 significantly inhibited the migration of A375, HT144 and B16F1 cells (−60%, $P < 0.001$; −30%, $P < 0.05$; −30%, $P < 0.05$, respectively), whereas the corresponding scramble peptide had no effect on cell migration.

Altogether, these results indicate that the LRR 9 motif sequence of lumican is responsible for the inhibition of the melanoma cell migration. We propose the name of lumcorin (fragment of lumican core protein) for this peptide.

Complementary experiments showed that lumcorin does not influence cell adhesion on lumican (Supplementary material, Fig. 1) or type I collagen (Supplementary material, Fig. 2). On the other hand, it was able to inhibit cell migration through collagen-coated Transwell® membranes (Supplementary material, Fig. 3).

To address the question of the function of LRR 7 and LRR 8 domains, we performed additional migration assays comparing lumcorin effect to LRR 7-derived peptide (peptide 7) and LRR 8-derived peptide (peptide 8). Results are shown in Supplementary material, Fig. 4. Peptide 7 exhibited high hydrophobic properties and aggregated melanoma cells in a non specific way. Therefore, it was not suitable for our experiments. Peptide 8 did not decrease

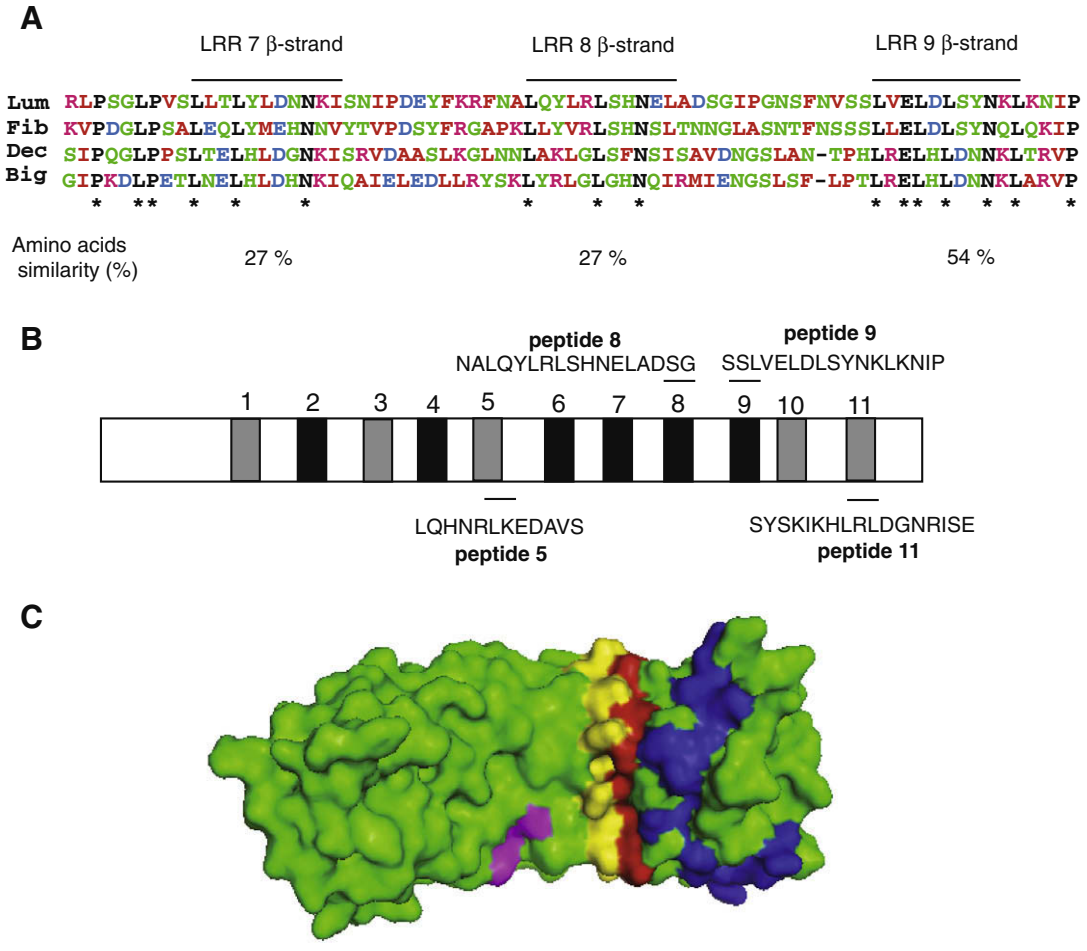

Fig. 3. Lumican amino acid sequence analysis. (A) Alignment of the partial protein sequences of four human SLRPs (lumican (Lum), fibromodulin (Fib), decorin (Dec) and biglycan (Big)) including the LRR 7–9 domains. LRR consensus sequences are indicated by stars and identical amino acids by black letters (blue: acidic amino acids, magenta: basic amino acids, red: small (small and hydrophobic) amino acids, green: remaining amino acids). The amino acid sequence similarity (%) between the four SLRPs for each LRR consensus motif is indicated below the sequences. The analysis was done using ClustalW2 software accessible at the European Bioinformatics Institute (http://www.ebi.ac.uk). (B) Sequences and localization of the different synthetic peptides used in this study. The amino acid sequence and its location are represented for each peptide. (C) View of the concave side of lumican molecule, modeled after decorin and biglycan crystal structures (PDB # 1xku and PDB # 2ft3). Part of peptide 5 and peptides 8, 9 and 11 residues are shaded in pink, yellow, red and blue respectively.

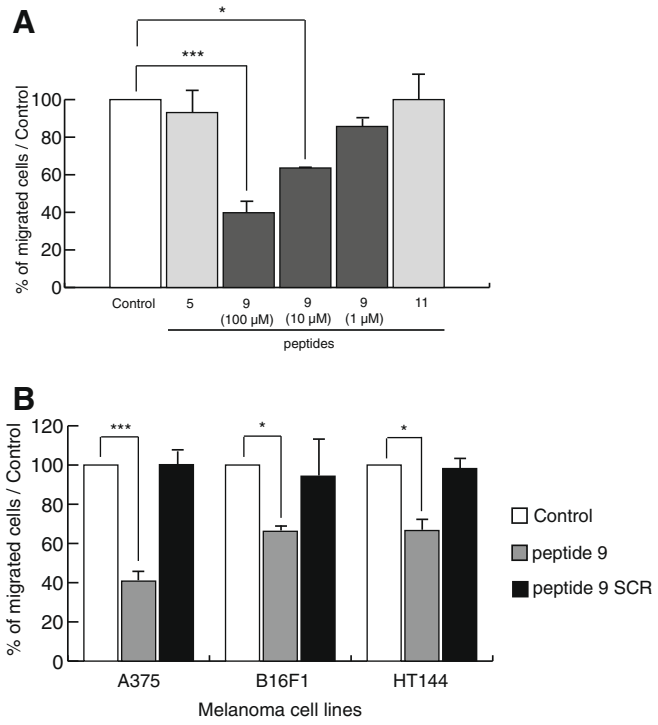

Fig. 4. LRR 9 motif inhibits melanoma cell migration. (A) Effect of the three synthetic peptides (5, 9 and 11) on A375 melanoma cell migration. Cells (5×10^4 per dish) were pre-incubated for 2 h at 37 °C in suspension with either the peptide 5 or 11 at concentrations 0 (control) or 100 μM or the peptide 9 at a concentration of 0 (control), 1, 10 or 100 μM (grey columns) and then seeded for 24 h at 37 °C on BSA-coated Transwell® membranes. (B) Effect of peptide 9 (100 μM) on the migration of different melanoma cell lines. Cells were incubated with either the peptide 9 or peptide 9 scramble (9 SCR) (100 μM) or without peptide (control) before being seeded on Transwell® membranes. The migration was measured as described in Fig. 1. ANOVA performed with the Fisher's test confirmed that the groups of data were significantly different for one group to another one (Fig. 4A, $F^5_{84} = 15.93$, $P < 0.001$; Fig. 4B, $F^2_{42} = 9.1$, $P < 0.001$, $F^2_{15} = 6.76$, $P < 0.01$; $F^2_{15} = 39.8$, $P < 0.001$ for A375, B16F1 and HT144, respectively). ∗, significantly different from the control by unpaired Student's test ($^*P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$).

significantly B16F1 cell migration compared to control. A375 cell migration was inhibited by peptide 8, but to a lesser extent than lumcorin.

4. Discussion

Tumor cell migration is an important process during melanoma progression, leading to invasion and metastasis. ECM macromolecules may be involved in the control of this process [19]. Lumican, a component of the ECM, may be considered as an anti-tumor molecule, since it is able to decrease melanoma progression in vivo [12]. Lumican is located in the dermis and in the peritumoral stroma of malignant melanoma, suggesting a defense mechanism against melanoma progression [11]. Here, we demonstrate that the migration inhibition is due to a specific sequence of the lumican core protein located in the LRR 9 domain. We propose to give it the name of lumcorin (fragment of lumican core protein).

In SLRPs, protein binding sites are located in the concave side of the molecule. This side is formed by parallel β-sheets, formed by the LRR motif sequences LxxLxLxxNxL [5,20]. For this reason, we focused on LRR motifs to determine the active site of lumican responsible for migration inhibition. The alignment results of the four main SLRPs suggested the LRR 9 motif as a potential target. Lumcorin affected cell migration only without any alteration of cell adhesion on lumican or type I collagen substrata (Supplementary material, Figs. 1 and 2). The fact that lumcorin did not compete with the lumican protein in the adhesion assays, indicates the presence of distinct site(s) in lumican for cell adhesion and cell migration.

Collagen is the major component of the extracellular matrix. As lumican is known to interact with type I collagen [10], an interference could occur on the inhibitory effect of lumican. Here, we observed that lumcorin inhibited the migration of melanoma cell, through collagen-coated Transwell® membranes (Supplementary material, Fig. 3), showing that the presence of collagen does not jeopardize the inhibitory effect of lumcorin on cell migration. We previously showed that the presence of type I collagen did not affect the remodeling effect of lumican core protein on melanoma cell cytoskeleton [21].

Lumcorin displayed different migration inhibitory efficiencies, as compared to lumican, depending on the cell line. Particularly, it decreased the migration of B16F1 and HT144 cells by 30% only whereas lumican core protein inhibited the migration of these cells by about 60%. This might mean that other lumican domains, especially LRR 7 and LRR 8 might be involved in the inhibition of cell migration. Results that we obtained with peptide 8, however, did not confirm this hypothesis, at least for B16F1 cells (Supplementary material, Fig. 4). We cannot exclude an effect of peptide 7 since we were not able to test it in the migration assays, due to its highly hydrophobic properties. Another explanation could be that adhesion receptor expression might be different between the three cell lines. We observed that the adhesion of B16F1 cells on lumican was three times higher compared to A375 cells (Supplementary material, Fig. 1). We previously showed that adhesion of melanoma cells to lumican may impair cell migration [13]. In contrast, A375 cells could have more receptors for lumcorin than B16F1 and HT144 cells. Further studies will be necessary to test this hypothesis.

Tumor progression could also be controlled by induction of apoptosis and lumican has been reported to facilitate the induction of the pro-apoptotic receptor Fas [22]. In our model, the inhibition of melanoma migration by lumican was not due to a pro-apoptotic effect. On the contrary, a rapid rearrangement of actin filament organization was observed when melanoma cells were grown on lumican [23]. Previous data from our laboratory suggest that this interaction could induce a reorganization of focal adhesions, leading to an immobilization of melanoma cells [21]. This could contribute to slow-down melanoma progression.

Decorin and biglycan, two other members of the SLRP family, were also described as anti-tumor molecules. We suggest that these SLRPs might have similar anti-migratory properties through their own LRR 9 motifs. Particularly, it could be interesting to focus on the fibromodulin LRR 9, which differs from lumcorin by four amino acids only.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.febslet.2009.08.012.

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